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HAPLOINSUFFICIENCY OF *RAI1* AND ITS EFFECT ON *BDNF* EXPRESSION

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at
Virginia Commonwealth University.

by

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December, 2010

Acknowledgements

First, I would like to give a big thank you to Dr. Sarah Elsea for being a great mentor throughout my master's career. She has guided me in the right directions, and showed an extraordinary willingness to help at any time. I thank her for supporting me and my research project and always being available to discuss ideas and suggestions. She has always listened carefully to me and showed an interest in my ideas. Above all, she has helped me in becoming not only a better scientist, but also a better person. I would also like to thank the Elsea lab for their support. I thank them for being wonderful lab partners and co-workers. I would especially like to thank Stephen Williams for always being there to lend a helping hand. When I first arrived in the lab with no knowledge of genetics research technology, he helped me to understand and utilize different molecular techniques. He was also readily available to offer suggestions and give feedback on my ideas. I would also like to thank the present and past lab members: Sureni Mullegama, Kristie Schmidt, Eri Kamura, Ria Vjas, Brooke Burns, Anam bashir, Jesus Cruz, and Catie McConnell. They were more than just lab members – they provided important support for my work.

I would like to thank Dr. Jolene Windle and Dr. Andrew Davies for being on my committee and always being willing to give more suggestions and ideas, and to help me to step back and see the bigger picture. I also would like to thank Dr. Rita Shiang for being a great advisor and mentor throughout my time at VCU and always being available to talk and give advice on any issue. She encouraged and supported me every time I needed it.

I would also like to thank Ms. Manavi Johri for technical assistance, and Dr. Roy Sabo for help with the statistical portions of my work, as well as Dr. Robert Vann and Mary Tokarz for their assistance with the data collection portion of the ampakine study.

I would also like to thank my friends, Dyvia Srikanthan, Noran Aboalela, Anting Hsiang, John

Moore, Brian Cho, Hung Nguyen and Steven Ko for their wonderful support throughout my entire time at VCU. We have shared so many memories and unforgettable days in Richmond together.

Lastly, I would like to thank my family and my parents. I thank my parents for always supporting and trusting me. They have always encouraged me to pursue whatever I wanted to do, and have supported me financially, emotionally and in countless other respects. Without their support, I would not be who I am now. I want to let my parents know how much I love them and how much I appreciate their trust in me.

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Abstract

**HAPLOINSUFFICIENCY OF *RAI1* AND ITS EFFECT ON *BDNF*
EXPRESSION**

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Smith-Magenis Syndrome (SMS) [OMIM, #182290] is a congenital anomaly and mental retardation (MCA/MR) syndrome associated with deletion of chromosome 17p11.2 [1]. The clinical phenotype has been well described and includes minor craniofacial anomalies, self-injurious behaviors as well as sleep disturbances, speech delays, and obesity [1,2,3]. The incidence of SMS is estimated to be ~ 1:15,000 - 25,000 births [2,6]. Among SMS patients, ~90% are comprised of 17p11.2 deletions, while ~10% have *RAI1* mutations [8]. All 17p11.2 deletions associated with SMS include *RAI1* deletion [10]. *RAI1* is thought to function as a transcriptional factor although its cellular role is still unclear.

First, in order to better understand the role of *RAI1* as a transcriptional factor and its relation to SMS, we confirmed that *RAI1* regulates *BDNF* within an intronic region. This sequence was further narrowed down by utilizing the luciferase reporter assay. This test confirmed what was previously found using ChIP-chip assay and microarray analysis of *Rai1*^{+/-} mice hypothalami. Next, in order to evaluate the role of *Bdnf*, an ampakine drug was administered to the *Rai1*^{+/-} mouse model. A mouse model is a powerful tool for studying a specific gene. *Rai1*^{+/-} mice exhibit the SMS phenotypes of obesity, craniofacial abnormalities, reduced pain sensitivities, seizures and others. Many physical, neurological, and behavioral tests were performed on the mice to see if any of the phenotypes can be rescued. Interestingly, twice-daily injections of ampakine CX1837 restored the pain sensitivities in *Rai1*^{+/-} mice. The hot plate data suggest that *BDNF* potentially has a role in regulating the SMS phenotype of decreased pain sensitivity. In order to evaluate other genes that are altered as a result of the CX1837 ampakine drug, the whole brain's global gene expression was evaluated via microarray analysis. Two potential pain-related genes were identified to be upregulated due to drug administration, which could account for the pain phenotypes observed. One of the genes upregulated in treated mice was *Osm*, which is interesting because *Osm* is responsible for pain sensitivity. Further analysis is needed to confirm that an ampakine drug can potentially be used to treat SMS patients.

CHAPTER 1: Background and Literature Review of Smith-Magenis Syndrome and *RAI1*

Smith-Magenis Syndrome

Smith-Magenis Syndrome (SMS) [OMIM, #182290] is a congenital anomaly and mental retardation (MCA/MR) syndrome associated with deletion of chromosome 17p11.2 [1]. The clinical phenotype has been well described and includes minor craniofacial anomalies (brachycephaly, prominent forehead, broad nasal bridge), self-injurious behaviors (head - banging, wrist - biting) as well as sleep disturbance, speech delay, and obesity [1,2,3]. Most patients with SMS have a large and common deletion of ~4 Mb, but many other affected individuals have smaller deletions involving 17p11.2 [4,5]. The incidence of SMS is estimated to be ~ 1:15,000 - 25,000 births, which is thought to be an underestimate, due to either subtle clinical features seen early in life or the manifestation of similar phenotypes with other syndromes such as Down syndrome and Prader-Willi syndrome [2,6].

***RAI1* and its role in SMS**

The 17p11.2 region contains many repetitive elements, including low copy repeats and three highly conserved regions called SMS-REPS (proximal, middle, distal REPS) [7]. Within SMS patients, ~90% of the cases account for 17p11.2 deletions, while ~10% have *RAI1*

mutations [8]. Among the 90% of 17p11.2 deletion cases, 70% of patients have a common deletion of ~3.5 Mb caused by homologous recombination between the distal and proximal REPs [9]. The other remaining 20% have atypical deletions (smaller or larger), with a minimum deletion of 700 kb in size [10,11]. All 17p11.2 deletions associated with SMS include *RAI1* deletion [10]. Although most of the SMS cases are *de novo*, one parental mosaicism case has been found [12,13].

RAI1 (GeneBank AY172136, AJ271789; OMIM*607642) maps to the central portion of the SMS critical interval, adjacent to *SREBF1* [12]. The gene contains 6 exons, generating ~8.5 Kb mRNA and 1906 amino acid proteins, with a molecular weight of ~203kD [12,14]. The protein contains two putative bipartite nuclear localization signals (NLSs), polyglutamine and polyserine tracts, and a PHD/zinc finger domain at the C-terminus [14,15]. *RAI1* mRNA is ubiquitously expressed throughout the tissues, and relative to the other tissues, the heart and brain tissues have stronger expression by two- and threefold, respectively [14]. Northern blot showed similar expression levels of *RAI1* in all the regions with the exception of the corpus callosum, where no expression was observed [14]. In mice, it has been shown that *Rai1* is mainly expressed in neuronal brain structures during development and adult life and shares an 84% homology with human *RAI1* as well as similar expression patterns [14,16]. There are a few differences between human and mouse *Rai1*. For example, in humans, there are a total of 10 to 18 glutamines, while in mice, this polyglutamine repeat is only 4 amino acids long [14]. Another difference is that the polyserine domain is present only in the human protein and not in that of the mouse [14]. Also, there is a human-specific carboxy-terminus [14].

Bioinformatic analyses suggest that *RAI1* is likely to play a role as a transcriptional regulator, however, its cellular and developmental role is still unclear [15,17]. *RAI1* shares more

than a 50% similarity with TCF20, a transcriptional cofactor, and these two genes have a similar structure [18]. TCF20 is involved in growth and neurobehavioral regulation [15].

SMS Clinical Overview

SMS phenotypes are clinically recognized via physical, developmental, neurological, and behavioral features [6]. These features of SMS become more pronounced and distinguishable with advancing age [19]. Also, significantly decreased fetal movement is noted in 50% of affected pregnancies [20]. Difficulty feeding is pronounced in early infancy, which ultimately leads to failure to thrive [20].

Physical features include craniofacial and skeletal anomalies such as high forehead, small nose with depressed nasal bridge, micrognathia, long philtrum with thin upper lip, broad square-shaped face, and ear anomalies [3,6]. The most common physical findings include brachycephaly with flat midface, broad nasal bridge, brachydactyly, and short stature [2]. Obesity is also commonly seen in teens and adults, along with broad chests [6]. Observed skeletal anomalies include brachydactyly, polydactyly, and fifth-finger clinodactyly, to name a few [1,3,21,22,23].

The majority of SMS patients (96%) have delayed speech, with or without hearing loss [6]. Most SMS patients have mild-to-moderate mental retardation (IQ range= 20-78) [1]. Individuals with SMS also have poor short-term memory but stronger long-term memory and perceptual closure [1].

Behavioral phenotypes are one of the major concerns for SMS patients, as aggression, disobedience, distraction, and self-injurious behaviors are frequently observed [6]. It is also worth noting an observed decrease in sensitivity to pain in some cases, which may have play a

role in self-injurious behaviors. Self-injurious behaviors include head banging, skin picking, and wrist biting as well as onychotillomania (fingernail and toenail picking) and polyembolokoilomania (object insertions into bodily orifices) [2,6,24]. Some SMS patients are diagnosed with ADHD, OCD, autism, and hyperactivity [25]. Other neurological features include abnormal gait and balance problems. Seizures are seen in 30% of patients [1,8].

Sleep disturbance is one of the earliest diagnostic indicators of SMS, and has been reported in 75-100% of SMS cases [1,6,26]. The phenotypes include difficulties falling asleep, diminished REM sleep, fragmented and shortened sleep cycles with frequent nocturnal and early-morning waking, and many others [6]. Inverted circadian rhythms of melatonin are responsible for these abnormal sleep patterns [25,27]. All children with SMS who were tested displayed a phase shift in their circadian rhythm with irregular levels of melatonin during the day. Melatonin reached peak levels at 12 PM \pm 1, compared to a normal peak time of 3:30 \pm 1:30AM [25].

Mouse model for SMS

Mouse models are particularly useful in uncovering the functions of genes and studying human diseases. The human chromosome 17p11.2 is highly conserved and syntenic to the mouse chromosome 11, which makes it feasible to establish a mouse model for SMS [4]. *Rai1*^{+/-} (Heterozygous *Rai1*-mice) were created by insertion of an *Escherichia coli lacZ* coding sequence and a neoR expression cassette into *Rai1* exon 2 while deleting 3910 bp nucleotides [28]. This is a strong model for studying the function of *Rai1 in vivo*, as it results in a truncation of the *Rai1* protein, eliminating all the nuclear localization regions and the PHD domain [28]. *Rai1*^{+/-} mice were viable and fertile and weighted slightly less than the wild-type littermates until 2 weeks of

age [28]. However, they become significantly obese by the age of 20 weeks [28]. In addition, 18% of the *Rai1* +/- mice exhibit craniofacial anomalies including hyperthelorum (broader distance between the eyes) and shorter and broader snouts [29]. These anomalies were found to be due to malformation of the craniofacial skeletal elements, as indicated by skeletal analysis [28].

For *Rai1*^{-/-} mice (Homozygous *Rai1*-mice), significant embryonic lethality and postnatal growth retardation are observed [28]. Overall, the mouse model successfully replicates phenotypes seen in human SMS patients.

Chapter 2: Confirmation of RAI1 regulation of *BDNF*

Introduction

Mutation or deletion of *Retinoic acid induced 1 gene (RAI1)* results in Smith-Magenis syndrome. However, its cellular and developmental role is still unclear and very little is known about the gene. Looking at TCF20, which shares more than a 50% similarity, RAI1 is likely a transcriptional factor [18]. Transcription factors can bind to nuclear DNA or other proteins, forming initiation complexes and starting transcription of other genes. Transcription factors can also repress transcription by blocking the enhancer binding regions. In an effort to uncover the function and role of RAI1, another student in our lab performed chromatin immunoprecipitation with microarray (ChIP-chip) (S.R. Williams, data not shown) to find if RAI1 binds to any chromatin fragments. The analysis narrowed the top 10 candidate genes to *SNRPN*, *UBE3A*, *CLOCK*, *MEF2A*, *BMP5*, *TRPC3*, *BCHE*, *CREM*, and ***BDNF***. In addition, microarray analysis on *Rai1*^{+/-} mice hypothalami [30], showed that there was alteration in *Bdnf* expression among many genes. Quantitative RT-PCR analysis of hypothalamic tissue confirmed that the *Bdnf* level was downregulated by 2.5 fold in *Rai1*^{+/-} mice compared to WT controls (B.M. Burns, data not shown). Taking these data together, testing whether or not *RAI1* regulates *BDNF* on a molecular level would be the appropriate next step.

Brain-derived neurotrophic factor (BDNF) is a second member of the “neurotrophic” family of neurotrophic factors, which was first isolated in the pig brain [31]. The *BDNF* gene has four 5’ exons (exons I-IV) with distinct promoters, and one 3’ exon (exon V) that encodes the mature BDNF protein [32,33]. The total of eight distinct mRNAs are transcribed predominantly in brain with some found in the lung and heart [33]. BDNF is synthesized as a preprotein which is proteolytically cleaved to release the mature form comprising of 120 carboxy terminal amino acids [33]. The highest levels of mRNA and constitutive BDNF protein are found within the hippocampus [34]. The BDNF protein is found to have a 50% amino acid identity with nerve growth factor (NGF) [35]. BDNF has been shown to support the development, survival, and differentiation of neurons but it is widely distributed in the central nervous system [36,37]. Altered expression of BDNF has been identified in depression, schizophrenia, and obsessive-compulsive disorder [36,38,39,40,41]. Further, haploinsufficiency of *BDNF* in humans is known to be associated with hyperphagia, obesity, and developmental problems [42].

Materials and Methods

PCR amplification of the *BDNF* regulatory region. The *BDNF* intron 1 element identified by ChIP-chip (Hg18, Chr11:27,680,656-27,681,712 Mb) was PCR amplified using Elongase (Invitrogen) following the standard protocol. The Primer sequences for *BDNF* element are as follows (2692 bp):

Forward: TGCCCGGTATGTACTCCTTC

Reverse: CAATTATGCCAGAGGCCAAT

Creation of plasmids.

BDNF long^{Luc} : The amplified PCR product was Taq treated and cloned into StrataClone™ PCR Cloning vector using the standard protocol provide by the manufacture(Agilent Technologies, Santa Clara, CA), creating a *BDNF^{Strata}* plasmid. The insert was confirmed by standard Sanger sequencing. The insert was removed by restriction digest with *KpnI* and *SacI* enzymes (New England Biolabs, Ipswich, MA). The resulting product was run on a 2% agarose gel for 30min and purified using Qiagen (Germantown, MD) Qiaquick gene extraction kit according to the manufacturers's standard protocols. *KpnI* and *SacI* enzymes were then used to “prepare” the pGL3pro vector (Promega Corp, Madison, WI) and the isolated insert from *BDNF^{Strata}* was directionally ligated into the pGL3pro vector to create *BDNF long^{Luc}* using standard protocols provided by the manufacturer (Promega Corp, Madison, WI).

BDNF Short^{Luc} : Using the *BDNF long^{Luc}* created above, an extra *KpnI* site within the *BDNF Short^{Luc}* sequence was created by switching nucleotide A to T using a QuikChange™ Site-Directed Mutagenesis. After this, the nucleotide sequence was shortened to 792bp. This mutagenesis and cloning were done by Christine Bax in the Laboratory of Dr. Debbie Zies

(University of Mary Washington). The shorter form of the *BDNF* sequence is shown in Figure 1 as underlined.

Transfections. (Protocol adapted from Stephen Williams's Thesis, 2010) Human embryonic kidney (HEK293T) cells were maintained in 6-well dishes containing Dulbecco's modified Eagle's medium with 10% (v/v) FBS, 2 mM L-glutamine, and 100 µg/ml of penicillin/streptomycin (Invitrogen, Carlsbad, CA) at 37°C in a 5% CO₂ incubator. The cells were counted using the trypan blue exclusion method to ensure >90% viability. Transfections with *pUC19*, *psv1-Gal*, *BDNF^{Luc}* and *RAII^{Flag}* were performed using LipofectamineTM 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Briefly, ~5 x 10⁵ cells were plated in 2.0 ml of growth medium without antibiotics 24 hours prior to transfection. A total of 4 µg of plasmid DNA, using pUC19 plasmid as “filler” DNA, were diluted in 250 µl OptiMEM® Reduced Serum Medium (Invitrogen, Carlsbad, CA). Similarly, 10 µl of LipofectamineTM 2000 was diluted in 250 µl of OptiMEM® Reduced Serum Medium, mixed well, and incubated for 5 minutes. After incubation, diluted plasmid DNA was mixed with diluted LipofectamineTM 2000 to a total volume of 500 µl and incubated for 20 minutes, then plasmid:Lipofectamine complexes were added to each well and mixed by rocking. Cells were incubated at 37°C in a 5% CO₂ incubator for 24 hours.

Luciferase reporter assay. (Protocol adapted from Stephen Williams' Thesis, 2010) After plasmid DNA transfection and 24 h incubation, cells were washed with 2 mL DPBS (Invitrogen, Carlsbad, CA) and Tropix Glacto-LightTM (Applied Biosystems, Bedford, MA) for which the standard protocol was used. Briefly, 250 µl of lysis solution was added to each well of the six

well plate and the plate was scraped until all cells were detached. Lysates were collected and centrifuged at 12,000RPM for 2min to pellet cell debris. Next, of the resulting supernatant 50 μ l was transferred to 4 wells of a 96 well white luminometer plate. Two wells were treated with 70 μ l diluted Galacton® substrate (1:100, Galacton:Reaction buffer diluent) (Applied Biosystems, Bedford, MA) and incubated for 30min. To the 2 wells that contained the diluted Galacton® substrate 100 μ l of Accelerator(-II) (Applied biosystems, Bedford, MA) was added. To the 2 wells that did not contain the diluted Galacton® substrate, 100 μ l of Steady-Glo® Luciferase substrate (Promega Corp, Madison, WI) was added. Each well was read using the Wallac 1420 VICTOR2™ Luminometer (PerkinElmer, Waltham, MA) on a maximum linear scale.

Calculation of relative luciferase activity. (Protocol adapted from Stephen Williams's Thesis, 2010) Relative luciferase activity from each individual transfection was calculated by dividing the average number of light units read from the wells containing the Steady-Glo® Luciferase substrate by the average number of light units from the wells containing the Galacton® substrate and Accelerator(-II). The equation ($\frac{\text{"Luc"}}{\text{"#-Gal"}} = \text{Relative luciferase activity}$) was used. Wells containing pUC19, psv#-Gal, and $BDNF^{Luc}$ were used as baseline for luciferase activity. Each experiment was performed independently at least three times.

Statistical analysis. P-values were generated by averaging relative luciferase activity from each independent study and performing a two-tailed student's t-test. Standard deviations were generated using GraphPad.

Results

Using a ChIP-Chip assay, a *BDNF* intron 1 region to which RAI1 putatively binds was identified. This *BDNF* region particularly maps to Chr11:27,680,656-27,681,712. Taking this information, primers were designed to amplify this region (Fig. 1) and cloned into a StrataClone TA cloning vector, followed by subcloning into pGL3-Promoter vector (*BDNF long^{Luc}*). The pGL3-Promoter vector is useful as it contains a SV40 promoter upstream of the luciferase gene providing baseline luciferase activity. HEK293T kidney cells were then utilized for transfection, as they have high transfection efficiency, and *RAI1* is highly expressed in kidney cells. The cells are co-transfected with RAI1a-GFP or RAI1c-GFP (Fig. 2) with *BDNF long^{Luc}* as shown in Materials and Methods. The relative luciferase activity was measured and compared between 1) *RAI1^{Flag}, pUC19, psv!-Gal*, 2) *RAI1^{Flag}, BDNF long^{Luc}, psv!-Gal*, 3) *BDNF long^{Luc}, pUC19, psv!-Gal*, and 4) *psv!-Gal* only. Each time, the transfection was performed in duplicate, and luciferase activity was measured in duplicate as well. At least 3 runs were done for each isoform. The luciferase reporter assay was utilized to see if there was any alteration of expression, indicating whether *RAI1* regulates by binding to this sequence. As shown in Figure 3., there is ~2 fold changes in relative light units when *BDNF long^{Luc}* was co-transfected with *RAI1* (isoform a). However, co-transfection of *RAI1* (isoform c) with *BDNF long^{Luc}* did not show any alterations in luciferase activity (Fig. 3). While RAI1 isoform a has 1906 amino acids, RAI1 isoform c only has 966 amino acids and lacks both a RAI1 bipartite nuclear localization signal and PHD domain, which are thought to be important for transportation of RAI1 into the nucleus for proper transcription to occur (Fig. 2). Therefore, this result was not surprising. A schematic diagram comparing RAI1a and RAI1c is shown in Fig. 2. Overall, the result shows that RAI1 binds to the cloned *BDNF* regulatory region, and regulates its transcriptional activity. The next question that

arises is whether it is possible to narrow down the binding sequence.

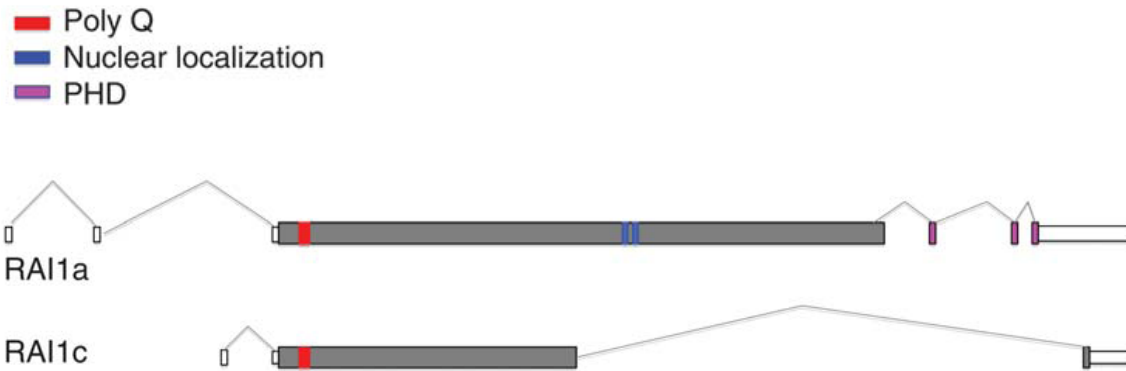
Following this question, the shorter form of *BDNF* (*BDNFshort^{Luc.}*) was created by removing 1809 bp, thereby resulting in the 780 bp sequence, as shown in Materials and Methods. This form was utilized to potentially further narrow down the *BDNF* regulatory binding sequence to which RAI1 (isoform a) binds. Interestingly, the reporter system expression level retained a ~ 2 fold increase when the *BDNFshort^{Luc.}* was co-transfected with RAI1a. This suggests that the RAI1 binding site/regulatory sequence is still present in the *BDNFshort^{Luc.}*.

With this information together we have confirmed that RAI1 has a direct binding in the *BDNF* intronic region, and we have further narrowed down its sequence to Chr11:276,648,82 - 276,656,73. Now, it is our goal to uncover the role *BDNF* plays in SMS phenotypes.

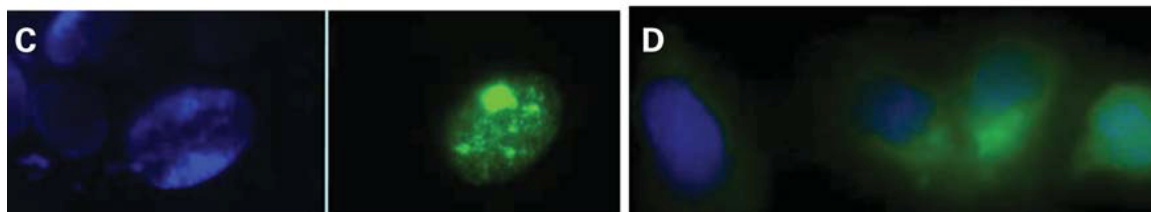
TGCCCCGGTATGTACTCCTTCtggtctgcagcaaagaagttaattattgatagtggaattgcatggcgggaggttaactcg
caccatcagcgcgagaagctccatttgatctcggcagaggcagggagattcatgctagttcgccggggggagcggcagcgagagcagc
cctctccgcgggtgaattgggaaagtgggtgggagtcacagagagggtccacgggtgccttgacgtgcgtgtcatatgatacctccgtgc
tcgaaatagacactctagtgcacgaattaccagaatcaaaattcagcgcatttaaaatgatacatctttattagaagagttccgttcaggga
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caattcagataaccatgatgaatcacacctcttaacacttgtaagcatttccaaactttctgatttgtgattatagcattgccaatactg
tatgcgaccagcttaaaacatgtacttatttgccttaaaagtacatcttacattcaaaaacaatgaagccaagataaaacaaaattgt
accagtgccttgcatggaattctaaatattatttttatttgccttaattcaggtagcatatagagataattttggaggtctaaaatcaaa
cattttacaaaattatttccctatttggggcaaggatgatgctgagaatatagagcatgcatttcttcttcttcttaaaaATTGGCC
TCTGGCATAATTG

Figure 1. Amplified *BDNF* long sequence and short sequence with RAI1 binding site.
Highlighted region shows sequence from ChIP-chip analysis (Chr11:27,680,656-27,681,712).
Bold and underlined sequence is shows sequence of *BDNF* in *BDNF^{short}^{Luc}*.

A.



B.



Source from Burns et al., 2010

Figure 2. Schematic diagram of RAI1a and RAI1c structures. A. Predicted gene structures of RAI1 isoforms are shown. RAI1a has 1906 amino acids, while RAI1c only has 966 amino acids which lacks nuclear localization and PHD domains. B. DAPI staining of nuclei transfected in HEK293 with RAI1a-GFP shows localization to the nucleus while RAI1c-GFP shows localization to cytoplasm.

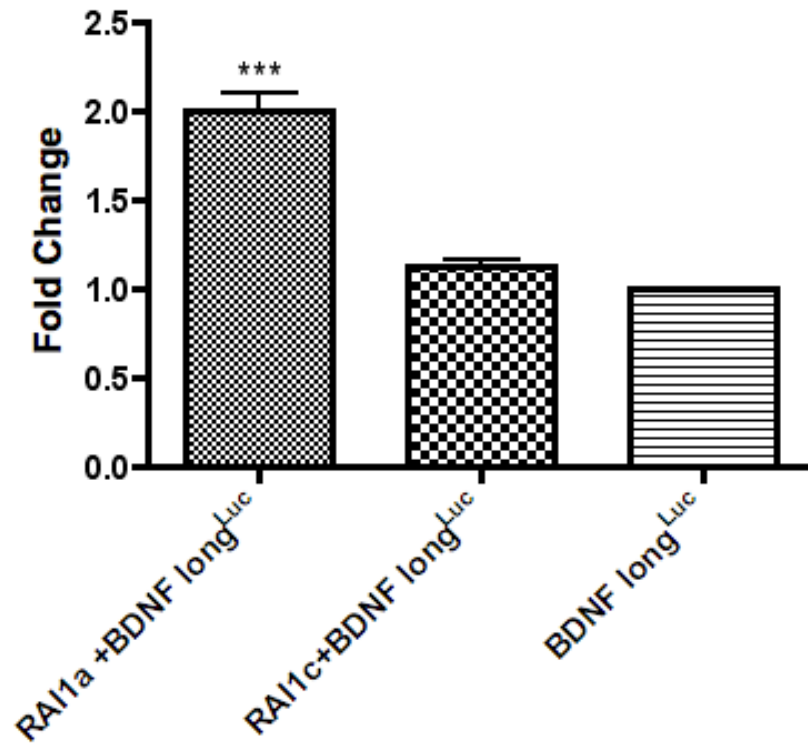


Figure 3. *BDNF* expression is regulated by *RAI1* isoform a. Luciferase activity of *RAI1* co-transfection with *BDNF*long^{Luc}. Negligible luciferase activity was detected from well containing *luc*-gal only. n≥3. *** p < 0.0001.

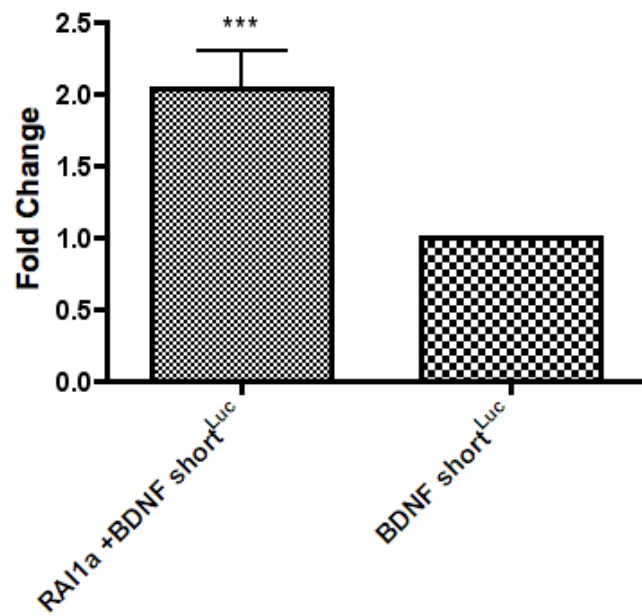


Figure 4. Narrowing down the *BDNF* region RAI1-a regulates. Luciferase activity of RAI1 co-transfection with BDNF long^{Luc}. Negligible luciferase activity was detected from well containing β -gal only. $n \geq 3$. *** $p < 0.0001$.

Discussion

Data presented here illustrate that one of the genes that RAI1 regulates is *BDNF*. The luciferase data clearly show that that RAI1 (isoform a) is positively regulating gene expression via the *BDNF* intronic region illustrated in Fig. 3 by binding directly to or within the subunit. While there is a two fold increase in luciferase activity with cotransfection of RAI1a and *BDNF^{Luc.}*, there was no alteration of luciferase activity with RAI1c. However, it is worthwhile to pay as much attention to co-transfection of RAI1c. By utilizing RAI1c, with its lack of NLS and PHD domain, it provides us with a confirmation that NLS and PHD are an essential part of RAI1. These data also suggests that RAI1c is likely playing a role elsewhere in the cell. Further investigation needs to be done on RAI1c.

In addition, using the shorter form of the *BDNF* intronic sequence (*BDNFshort^{Luc.}*) we were able to show that enhancement of transcription was maintained in the reporter assay. Since there was not much difference between the activities of *BDNFlong^{Luc.}* and *BDNFshort^{Luc.}*, it is apparent that the binding region is still present in the short form. Overall, the data successfully reveal two main conclusions: 1) RAI1 has a positive a role in regulating *BDNF* via the intronic region. 2) The region of *BDNF* to which RAI1 binds has been narrowed to ~800bp in *BDNF* intron 1.

BDNF is known to function in development, survival, and growth of neurons [37,43]. With its highest expression of both mRNA and protein in the hippocampus, which is known to be the site of the long-term memory, BDNF is associated with learning and memory [44]. Many reports have also demonstrated decreased level of BDNF in certain neurological diseases [45]. Taking these facts together, it seems that the central role of BDNF is taking place in the brain. On the other hand, reduced expression of BDNF has been associated with obesity [42,46]. As

many of the phenotypes of SMS are not exclusive, the next asks will be to narrow down the role of BDNF in SMS phenotypes.

Overall, this study provides a piece of the puzzle toward understanding RAI1 regulation of BDNF. It would be wise to look at the other top candidate genes that were found in ChIP-chip analysis to see if any can be confirmed on a molecular level, which would reveal more about the cellular function of RAI1, and help us to discover more of the many roles of RAI1. I believe that one day, most of the pieces to the puzzle will be found, and that we will be able to connect the pieces together and have a much greater understanding of the role of RAI1 than what we do now.

Chapter 3. Exploring the effect of ampakine drug treatment on *Rai1*^{+/-} mice

Introduction

The previous study suggested that *RAI1* is likely regulating expression of *BDNF*. Also, *Bdnf* expression is significantly lower in the *Rai1*^{+/-} mice compared to WT mice. BDNF was first purified in a pig brain based on its survival promoting action on dorsal root ganglion [31]. BDNF is known as an important regulator of synaptogenesis and synaptic plasticity mechanisms underlying learning and memory in the adult CNS. Another important role for BDNF in adulthood appears to be as a central modulator of pain [47]. The perception of pain implicates the activation of peripheral (cutaneous, muscular, articular or visceral) nociceptors, the generation of a nerve signal and the transmission of this signal to the somatosensory cortex. Rats subjected to the ligation of a spinal nerve (SNL) exhibited a hypersensitivity to thermal and mechanical stimuli that was reduced by intrathecal administration of BDNF [48]. Another study has shown that BDNF is required for the early postnatal survival of nociceptors [49].

BDNF is also known to suppresses food intake [50]. Furthermore, it has been reported that BDNF regulates food intake and glucose homeostasis in genetically obese animal models [51]. In heterozygous *Bdnf*^{+/-} mice, with its BDNF gene expression, hyperphagic behavior and dramatic obesity were reported [52].

Most cellular actions of BDNF's are mediated by its high-affinity receptor, the

neurotrophic tyrosine kinase receptor type 2, or TRKB [53,54]. TrkB is abundant during development, but also widely distributed in the CNS of adult animals, suggesting a continuing role for BDNF in the adult nervous system [47]. Human patients with TRKB defects also exhibit hyperphagia and obesity along with impaired learning and memory [55].

The mouse *Bdnf* gene (GeneBank AY05907) contains eight 5' noncoding exons and one 3' protein coding exons [56,57]. In mice, *Bdnf* mRNA is expressed throughout development and differentially in adult tissues. Similarly to the human model, *Bdnf* mRNA and protein expression is found throughout the brain with the highest expression in the neurons of hippocampus [58,59,60]. Neuronal *Bdnf* expression is affected by many stimuli such as GABAergic and glutamatergic neurotransmission and membrane depolarization through calcium-mediated pathways [61,62]. Gene expression is controlled by multiple activity-dependent and tissue-specific promoters that produce a heterogeneous population of BDNF mRNA [56].

Homozygous knockout *Bdnf* mice $-/-$ lack these neurons [63]. Knockout of *Bdnf* $-/-$ knockout mice are lethal and fail to survive past 3 weeks, while heterozygous *Bdnf* knockout ($+/-$) mice exhibit various phenotypes, obesity being one of them [52].

There are numerous studies showing that ampakine drug treatment increases protein BDNF levels. Recent studies showed that elevation of BDNF expression can be achieved both in vitro and in vivo using ampakines [64,65,66]. Ampakines are a novel neurolpharmacological class of small molecules that trigger the AMPA-type of glutamate receptors and increase ligand-gated current flow [67,68,69]. These compaounds were originally derived from aniracetam [69]. Also, repeated treatment with ampakines can increase the efficiency of long-term potentiation in the hippocampus and facilitate memory process [70,71,72]. The potential significance of ampakines arises from its ability to readily cross the blood-brain barrier, improve short term

memory, and facilitate olfactory learning with no evident side effects [73,74,75].

Several studies have used ampakine to restore *Bdnf* levels in mouse models of diseases. In the Huntington disease knock-in mouse model, upregulation of BDNF with twice daily injections of ampakine showed rescue in synaptic plasticity and memory by means of normalizing the *BDNF* level [76]. This study suggested that ampakine treatment can be a potential strategy for chronic treatment of Huntington disease [76]. Also, another study showed that ampakine treatment improved *Bdnf* expression as well as respiratory function in a mouse model of Rett syndrome [77]. Furthermore, ampakines are under investigation as a potential drug treatment for Alzheimer's disease, Parkinson's disease, schizophrenia, ADHD and other disorders that involve mental disability and disturbances. Based on this information, it seems that ampakines are a great candidate drug for increasing the expression of *Bdnf* to potentially provide a partial rescue of phenotypes seen in *Rai*^{+/-} mice.

Materials and Methods

Mouse models, breeding, and maintenance. Mice evaluated in this study were C57Bl/6J *Rai1*^{+/-} animals bred from stock received from Jackson Laboratories (stock #005981). Due to poor in-house breeding of this line, the mice were outcrossed to FVB/N to improve the number of offspring obtained. When the mice were being bred or in feeding studies, they were given access to a higher fat (9%) Lab Diet, 5P06 Prolab RMH 2000, and water. An automatic 12-hour light/dark cycle was provided at all times along with constant temperature (21C) and humidity (40%). The overall phenotype of these mice is muted in this mixed background, but the FVB/N x C57Bl/6J *Rai1*^{+/-} animals are overweight and have craniofacial anomalies similar to the B6 congenic strain originally evaluated.

Genotyping. Mice were genotyped by PCR analysis using template genomic DNA from mouse tails prepared as previously described (Girirajan et al., 2008). *Rai1*^{+/-} genotypes were confirmed using target allele and wild type primers as reported in B et al., 2005. Reactions were held at 94C for 5 min followed by 42 cycles of denaturing at 94C, for 1min, annealing at 60C and an extension at 72C, for 1 min, followed by a final extension at 72C, for 7 minutes. Reactions were held at 4C until use.

Ampakine drug preparation. CX1837 (Cortex Pharmaceuticals) was dissolved at a concentration of 0.25 mg/ml in 2-hydroxypropyl- β -cyclodextrin (HPCD) powder, with equal weights of water and 0.9% injectable saline as a 1:1:1 mixture, or 33% HPCD in water/0.045% saline. Once the HPCD powder was dissolved, the total volume was split in half. Ampakine CX1837 was added to half of the volume, and the remaining half was used as a vehicle. The

solution containing Ampakine CX1837 was then submerged in a sonicator heated to 37C and then frequently vortexed to aid solubilization. The resulting solution is a clear viscous liquid stored at 4C.

Ampakine injection. Each mouse was weighed weekly and given 1 mg/kg of ampakine CX1837 twice daily (10:00 a.m. and 5:00 p.m.). A vehicle solution was used at the volume of 0.25mg/mL. The solution was mixed well before use to get rid of any precipitation that had formed in storage. The volume of drug administered was altered once a week to account for weight fluctuations in the mice. The mice were anesthetized with isoflurane prior to intraperitoneal (IP) injection. Each mouse received the dose in the same order every day. The four treatment groups included wild type (WT vehicle treated, WT drug treated), *Rail*^{+/-} vehicle treated, and *Rail*^{+/-} drug treated.

Physical assessment and simple reflexes:

Sound orienting. Brief and sharp noise was made to the right and left of the mouse's ear. A turn in the direction of the noise source was noted.

Pupil constriction/dilation. A beam of light was shone in the direction of the mouse's eye. Constriction when the light was shown and dilation when the light was removed were noted.

Whisker response. A small paint brush was used to lightly brush the whiskers of the mice and their response was observed.

Eye blink. The tip of a clean cotton swab was used to approach the eye and determine if the mouse blinked in response.

Ear twitch. The tip of a clean cotton swab was used to touch the mouse's ears to see if the ear

twitches in response (both ears were tested).

Postural reflex. The mouse was placed in an empty cage that was tilted from side to side, and up and down, and the mouse's ability to maintain an upright position was observed.

Response to being picked up by tail. Mice were picked up by the base of their tail and held for 10 seconds. The mouse was then lowered to the ground. Limb extension upon lowering to ground, as well as head raising were measured visually.

Gaiting test. Each of the mouse's feet was colored a different color (non-toxic material). The mouse was then allowed to walk through a small tunnel on white paper. Stride, sway, and stance measurements (in cm) were made. Stride was defined as the distance between two rear paw prints, and sway as the distance between paws from side to side, and stance as the diagonal distance between two front or back paws.

Hot-plate test. The mouse was placed on an analgesic hot plate set at 60C and the time (in sec) it took for the mouse to display a common response (jump, raise paw, paw lick or paw shake) was measured. Unusual responses were also noted. The maximum trial time was 30 sec.

Tail flick test. The mouse was restrained gently with its tail placed in the groove of the tail flick apparatus. The 8V (6 amp) light automatically extinguished once the tail was removed. The maximum trial time was 10 sec.

Behavioral test:

Operant conditioning test. For operant responding tests, eight standard mouse operant conditioning chambers that were sound- and light-attenuated (MED Associates, St. Albans, VT) were used. Each operant conditioning chamber (18 x 18 x 18 cm) was equipped with a house light, two levers (left and right), and a recessed food pellet receptacle centered between the

levers. A hopper delivered food pellets into the receptacle. Fan motors provided ventilation and masking noise for each chamber. House lights were illuminated during sessions. A computer with Logic '1' interface (MED Associates) and MED-PC software (MED Associates) controlled schedule contingencies and maintained data. A food pellet was released every time the right lever was pressed. Mice were placed in the box and allowed to lever press for three 15 minute sessions throughout the course of the study. For the final test, mice were food deprived overnight and then placed in the chamber for 1 hour. Data was recorded as total number of lever presses. This testing was performed by Mary Tokarz.

Growth assessment:

Weight gain. Mice were weighed on a weekly basis to assess growth and weight gain or loss due to drug administration.

Feeding study. Food was weighed daily for a 5 day period. Mice were placed in isolation cages with a predetermined amount of normal chow (5% fat) and an unlimited amount of water. Food was weighed every 24 hours.

Gene expression studies:

RNA isolation. RNA was isolated using the Trizol reagent/chloroform method. The quality of isolated RNA is assessed by using a 260/280 and 230/260 absorbency ratio and the concentration was obtained using Nanodrop.

Real-time quantitative PCR. cDNA synthesis was performed using 4-5 ug of RNA, Invitrogen Superscript 2 reverse transcriptase and random primers according to the manufacturer's instructions. An ABI TaqMan probe for mouse mRNA expression was used to detect expression

of *Bdnf*. All samples were analyzed in triplicate in a 10 uL reaction on an ABI Prism 7500 Fast System with *Gapdh* as an endogenous control.

Results

I. Initial study- First round

The initial study was conducted with 6 mice; 3 females and 3 males. Of the 3 females, 2 were *Rail*^{+/-}, receiving vehicle and drug respectively, and 1 was WT, receiving vehicle. Of the 3 males, 2 were *Rail*^{+/-}, with both receiving drug and vehicle, and 1 was WT, receiving vehicle. The 3 female mice were born on the same day from the same mother, and the 3 male mice were born the week after. The ages of the females and males when the treatment was started was 9 weeks and 8 weeks, respectively. It is important to note that the mice used in the study were 50:50 mixed background progeny of C57Bl/6J and FVB/NJ, as pure *Rail*^{+/-} C57Bl/6J background mice have difficulty breeding and have reduced transmission of the mutant allele. The phenotypes observed in these mice are generally milder than the pure C57Bl/6J and varied greatly among the mice, (i.e. some had more pronounced phenotypes than others) and the behavioral characteristics of the mixed background mice were very different, as they were jumpier and more active than the pure C57Bl/6J mice.

Mice were evaluated for each test in the same order each time. Also, the mice were anesthetized under isoflourine before each treatment. It took a few minutes after the treatment for them to fully wake up and get back to normal. Therefore, all the tests describe below were performed a few hours after the drug treatment.

Neurological testing

Before any other tests were done, a general physical assessment was made and simple reflexes were tested. A note was made of any abnormal behavior after treatment was given. This was ensure that the drug does not have any negative side effects or a sedative effect.

Further, this examination ensured the accuracy of the results of any other testing. The mice were first tested for sound orientation, pupil constriction/dilation, whisker response, eye blink, ear twitch, postural reflex and response to being picked up by the tail. Regardless of their genotypes and whether they received the vehicle or drug, the mice all showed normal responses to these tests. The mice were able to turn in the appropriate direction when noise were made near their ears, and their ears twitched when touched with a cotton swab (data not shown). They blinked when a cotton swab was held near their eyes, and proper constriction/dilation were observed when a light was shown/removed (data not shown). When the cage was shaken up and down, as well as side-to-side, all mice were able to maintain their balance and remained upright (data not shown). When they were being picked up by the base of the tail, all of them raised their heads and reached for the ground upon lowering, which is the normal response (data not shown).

Gating was analyzed for each mouse. As described in Materials and Method, stride, sway, and stance length were measured. As shown in Fig. 5, there were no differences between the groups when they were measured (Fig. 5a,b,c). There was also no difference between the drug or vehicle groups for the drop test (Fig. 5d). Previous data collected from mice with a C57Bl/6J background indicated that the *Rail*^{+/-} mice have a significantly larger landing splay (S. Girirajan thesis, 2008), but the C57Bl/6J:FVB/NJ mixed background appears to mute this phenotype.

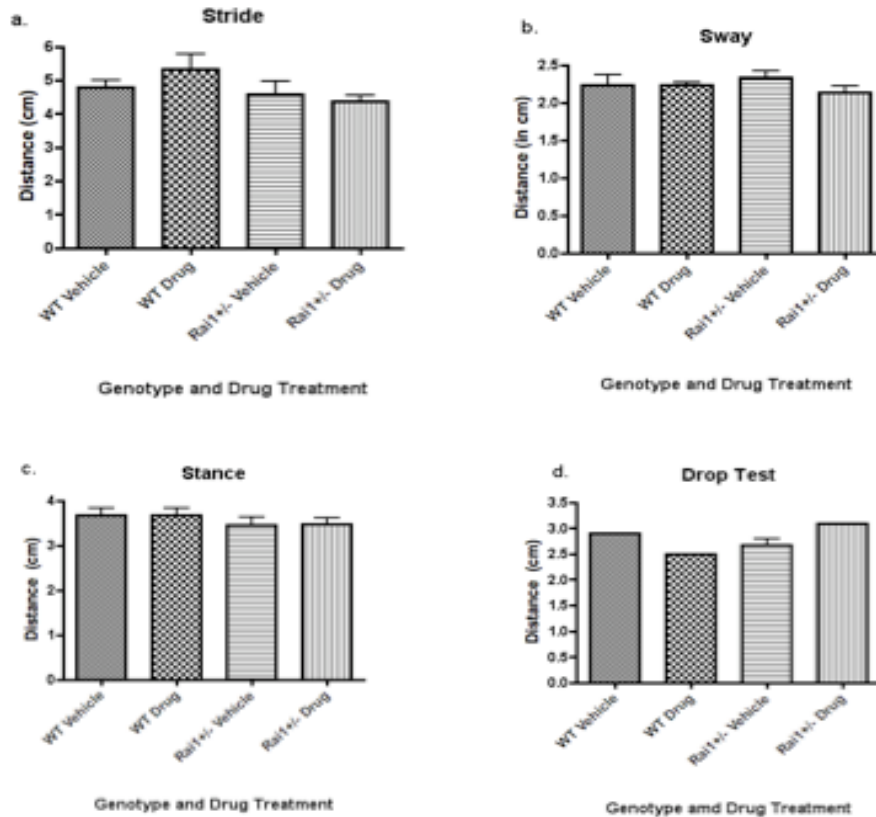


Figure 5. Gating tests and drop test of ampakine and vehicle injected mice. All data reported as mean±s.e.m.

Next, a hot plate test was performed to determine if the ampakine treatment could correct the delayed response to nociceptive pain documented in *Rai1*^{+/+} mice. As shown in Fig. 6a, the *Rai1*^{+/+} vehicle treated female stayed a longer time on the hot plate, which is consistent with what has been previously found (S. Girirajan's thesis). Interestingly, the drug treated female mice stayed on the hot plate for about the same amount of time as the WT vehicle treated female, indicating that the pain response is improved (Fig. 6a). However, there were no significant differences between the groups of male mice (Fig. 6b). As there were not enough mice to reach

any firm conclusions from this study, further study needs to be done to confirm the affect of ampakine treatment this phenotype.

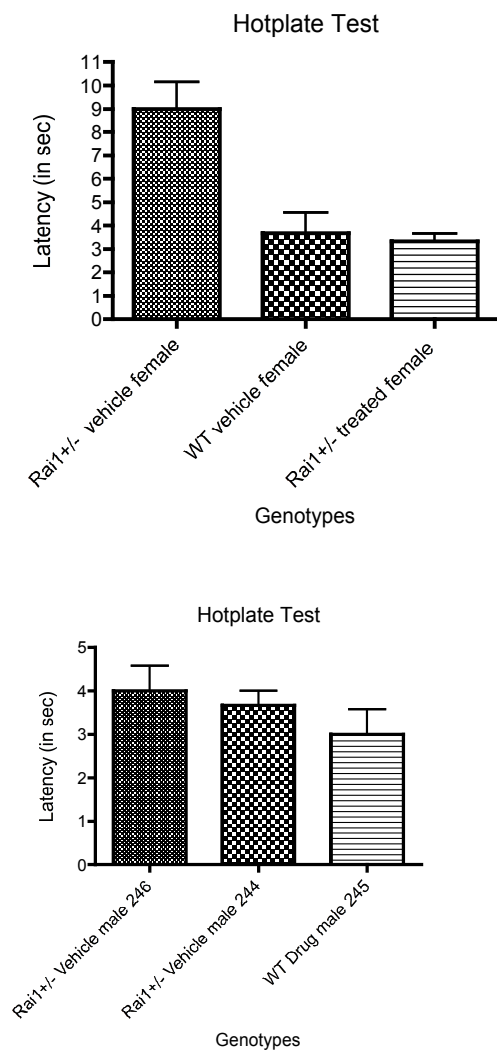


Figure 6. Initial hot plate test of ampakine and vehicle treated *Rai1*^{+/-} and WT mice. Maximum time for each mouse to be on the hot plate was 20 seconds. The data shows females and males separately.

Feeding Behavior

Over the course of the 8 week study, the weight of each mouse was recorded weekly and the growth curve was observed (Fig. 7a). Interestingly, drug treated *RaiI*^{+/-} female gained a great deal of weight, while the growth of vehicle treated *RaiI*^{+/-} female was comparable to WT vehicle treated female (Fig. 7a). On the other hand, the males weighed more than the females, and vehicle treated *RaiI*^{+/-} male mice seemed to gain more weight than WT male (Fig. 6b). Additionally, the mice's feeding behaviors were observed to access hyperphagia and its possible correlation with growth curves (Fig. 8). Pre-weighed food was given for 5 days, and the amount eaten was recorded. Two different sets of 5 days were observed. In order to normalize the amount that each mouse consumed, the daily amount consumed was divided by their weights. It was expected that the *RaiI*^{+/-} vehicle treated mice would consume more food relative to their weights. Interestingly, that was not the case. There were no differences relative to weight in the amount of food consumed between *RaiI*^{+/-} vehicle female and *RaiI*^{+/-} drug treated female, and no shifts in eating habits were found (Fig. 7). It is really interesting to see that *RaiI*^{+/-} drug treated female ate relatively similar amount with *RaiI*^{+/-} vehicle treated and yet still managed to maintain similar weight with WT vehicle treated. This suggests that there is some other metabolic pathway that is altered in *RaiI*^{+/-} that is responsible for their weight changes. Judging by these results, the drug does not seem to have any effect on the eating behaviors in these mice. It is important to note that vehicle treated and drug treated *RaiI*^{+/-} females did not start their growth curve at equal weights. Therefore, further study is needed with a greater number of mice to confirm these results.

Overall, the results of this initial study suggest that further study is needed if the hot plate test, weight gain test and relative feeding study are to confirm the effects of the drug.

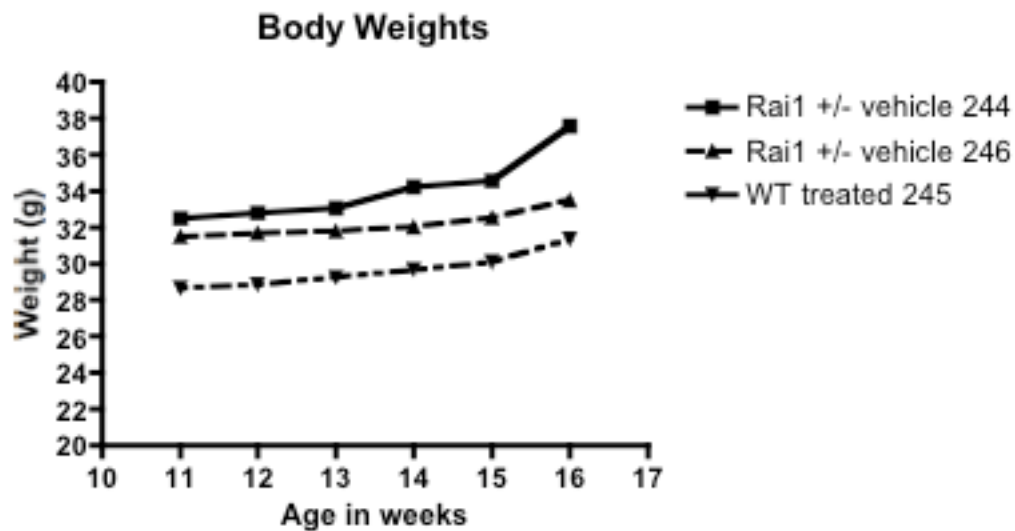
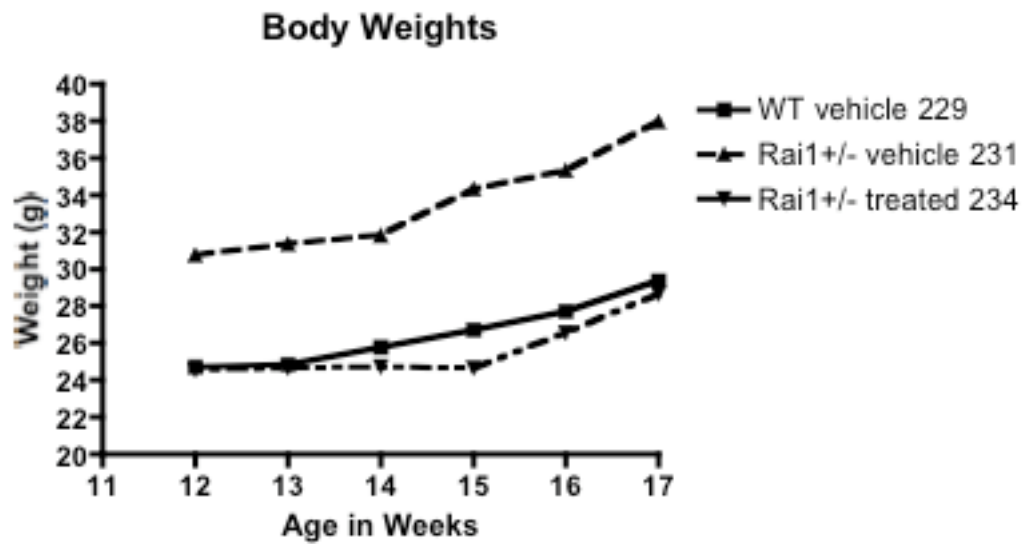


Figure 7. Weight of ampakine and vehicle treated Rai1+/- and WT mice. Mice were separated by gender and treatment group. Individual mice were plotted. Female group is on top and male group is at the bottom.

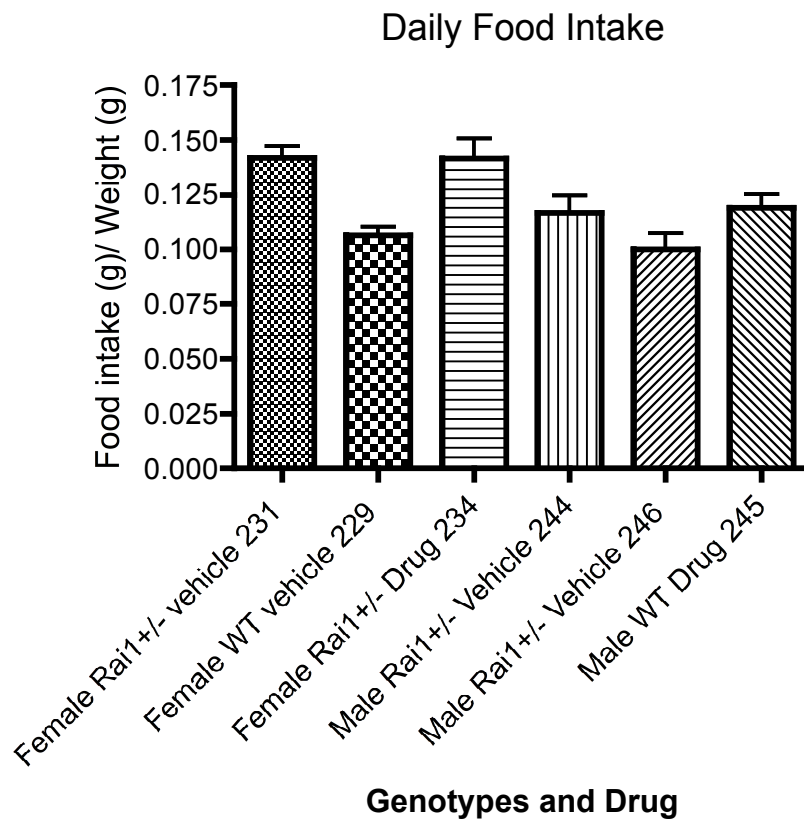


Figure 8. Initial food consumption of ampakine and vehicle treated *Rai1*^{+/-} and WT mice. Daily food intake was measured and divided by their weight of the mouse. The data show females and males separately.

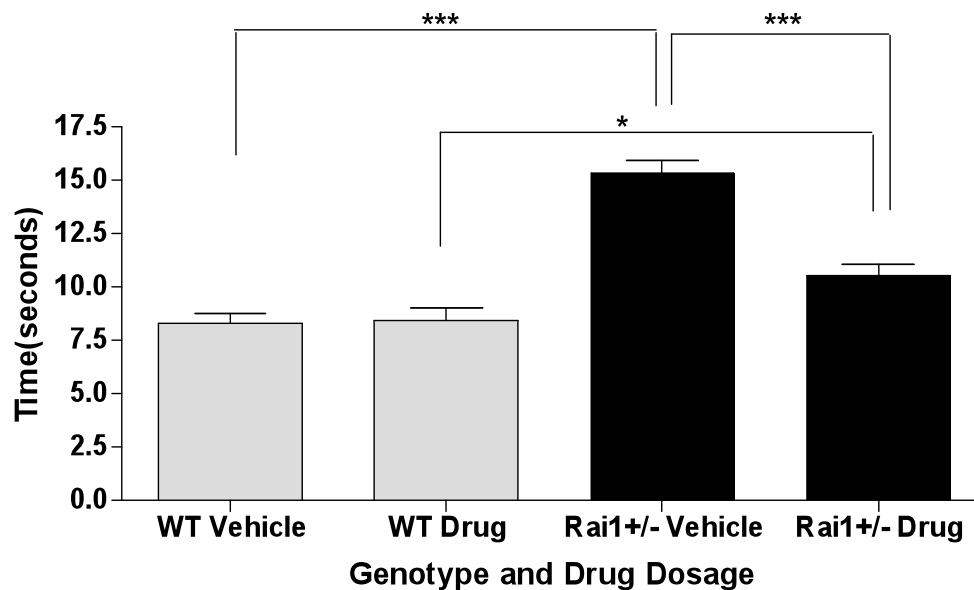
II. Second batch

For the second batch, 29 mice were used, of which 16 of them were *Rail*^{+/-} mice, and 23 were WT mice. Of the 16 *Rail*^{+/-} mice, 8 were in the vehicle group while the remaining 8 were in the drug treatment group. For the WT animals, 6 of them received the vehicle, and 7 were injected with the drug. Also, the mice were grouped into two age groups: 7 and 16 weeks. This round was conducted by another student in our lab (K. Schmidt, 2010).

Hot plate test.

As the drug seemed to have an effect on restoring the pain sensitivity of *Rail*^{+/-} mice, the hot plate test was repeated (K. Schmidt, 2010). As shown in Fig. 9, *Rail*^{+/-} mice can tolerate the hot plate a significantly longer amount of time than their WT littermates, regardless of gender ($p < 0.0001$, Fig. 9). When the *Rail*^{+/-} mice are treated with ampakines, the pain response in the *Rail*^{+/-} improves, and they showed a quicker pain response and spent less time on the hot plate than *Rail*^{+/-} vehicle mice. However, *Rail*^{+/-} drug treated mice spent significantly more time on the hot plate than the WT ampakine treated mice ($p < 0.0001$, $p = 0.0209$, Fig. 9).

With regard to the hot plate test, a few difficulties need to be mentioned. First, the time recording was done in a subjective manner. It is the observer's call to stop the watch when the mice seem to have a pain response. As mice tend to rear in response to pain, the data might not be the most accurate account of pain sensitivity. Further study needs to be done with more objective testing methods, for example, with an automated time recorder, in order to confirm these results.



Source from Kristie Schmidt's thesis, 2010

Figure 9. Hot plate test of ampakine and vehicle treated *Rai1*^{+/-} and WT mice. Maximum time for each mouse to be on the hot plate was 20 seconds. All data reported as mean \pm s.e.m. * $P < 0.05$, *** $P < 0.0001$.

Behavioral Testing.

Behavioral responses were evaluated in an operant test in which the mice learn to push a lever to receive food. It was predicted that if the ampakine treatment was decreasing the amount of food intake, that the *Rai1*^{+/-} mice would be less likely to press the bar for pellets than their vehicle treated *Rai1*^{+/-} siblings. Using a generalized linear model with poisson distribution, statistical analysis was performed to see if there was any differences in behavior between the groups. A trend suggests that drug treated mice, regardless of genotype, pushed the bar more frequently to receive food pellets. Further, when looking at treated mice specifically, the drug

treated mice both pressed levers significantly more often than vehicle treated mice, regardless of genotype ($p < 0.0001$, Fig. 10). This suggests that the drug has some effect on their behavior, as only the drug treated group showed a different response regardless of the genotype. Based on this data, different interpretations can be made. It could be that the drug is making the mice more hyper or possibly “smarter.” Note that there were 3 training sessions in which the mice learned that pressing the lever gives them food, and those data were only recorded during the 4th and the longest session. As the data shows a significant difference in the case of the drug treated group, it is possible that the treatment enhanced the treated mice' learning ability. It is important to note that the food eaten was not recorded and this is something that should be evaluated in the future.

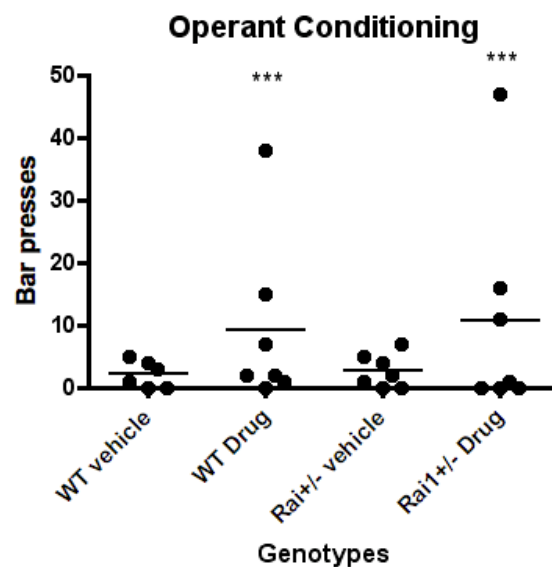


Figure 10. Operant test of ampakine and vehicle treated *Rai1*^{+/-} and WT mice. Operant data evaluated as bar presses/60 min, completed after 2 training periods. Statistics were done using a generalized linear model with tests of fixed effects, p -value < 0.0001 .

Growth assessment and feeding behavior

For the second batch, the weights were measured every day over a 2-week time period. The mice were separated into age and gender groups. Interestingly, the mice did not show significant changes in weight. There are few things to note, however. At day 1 of ampakine treatment, the 7 week old female *Rail*^{+/-} and WT drug treated mice had significantly different weights ($p = 0.0211$, Fig. 11). The 7 week old female *Rail*^{+/-} and WT vehicle treated mice ($p = 0.0056$, Fig. 11) and vehicle treated mice ($p = 0.0056$, Fig. 11) had significantly different weights. At day 1 of ampakine treatment, the 16 week old female *Rail*^{+/-} and WT drug treated mice had significantly different weights ($p = 0.0074$, Fig. 11). Male *Rail*^{+/-} and WT mice had no significant differences in weight at 7 or 16 weeks of age (Fig. 11). It is interesting to note that the trends for the vehicle and drug treated *Rail*^{+/-} mice in the 16 week old age group were the same. However, the data as a whole indicate that all of the mice in each of the dosage groups stayed at approximately the same weight throughout the study. Therefore, any effect the drug may have had on the growth curve is hard to conclude from this study.

Next, food intake was measured every day over a 2-week period. Each day, the amount of food the mice ate was measured and data was recorded as amount eaten divided by the weights (Fig. 12). Over the course of the 2-week period, when mice groups were compared, there was a significant difference in the *Rail*^{+/-} Drug and *Rail*^{+/-} vehicle treated ($p=0.0006$). However, there was no significant difference between *Rail*^{+/-} Drug and WT drug treated ($p=0.3254$). The significant difference seen in *Rail*^{+/-} drug mice can be due to the food crumbling seen in the drug treated *Rail*^{+/-} females. This behavior might have gave a false positive result as measurement of food cannot be accurate for it is difficult to account for the extra food seen at the bottom of the cages. Also, between the male groups, there were no

significant difference in any of the groups ($p=0.6893$).

Overall, restoration of pain sensitivity is observed consistently in both the first and second batch (Fig. 6, Fig. 9).

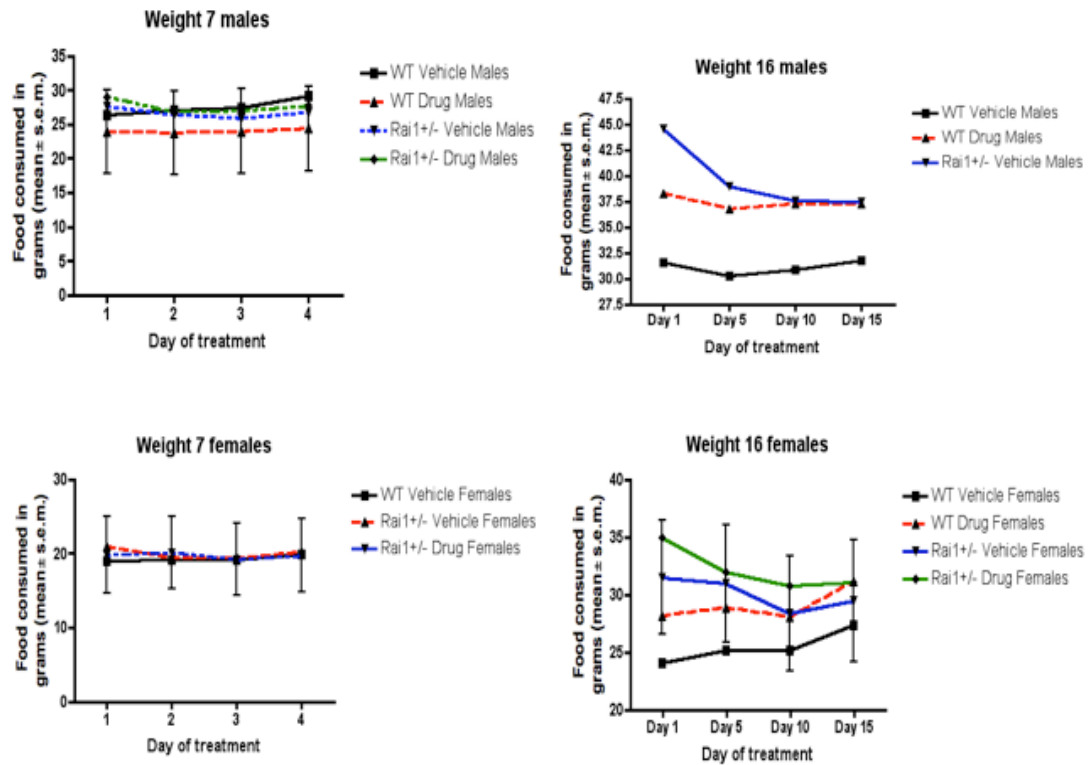


Figure 11. Weight of ampakine and vehicle treated *Rai1*^{+/-} and WT mice. Weights of 7 and 16 week old ampakine and vehicle treated female mice on top and male mice oat the bottom.

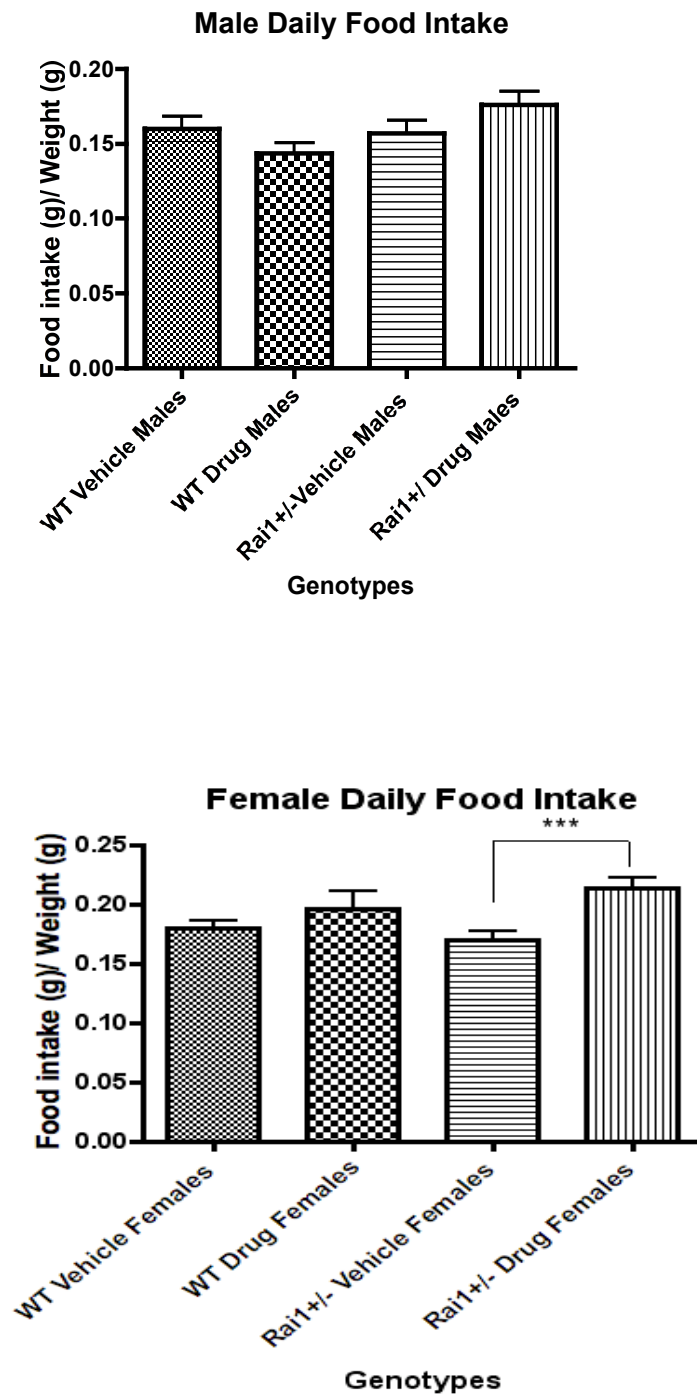


Figure 12. Food intake study. Age groups were pooled together as no significant differences were found between the groups. ***p=0.0006

Expression Studies in ampakine treated *Rai1*^{+/-} mice

After completion of the 2-week treatment period, all 29 mice were euthanized and their brain tissue was collected. For the RNA extraction, the brain without hypothalamus was homogenized. The mRNA expression level of *Bdnf* was evaluated to confirm that ampakine treatment increases *Bdnf* level.

The data show that ampakine treatment caused a subtle increase in *Bdnf* expression in WT mice as well, as there was ~1.2 fold increase in expression in the WT treated group. Also, the *Bdnf* level was increased (to ~80%) when compared to *Rai1*^{+/-} controls, which showed about 50% expression compared to the WT control, a result that is consistent with previous findings (B. Burns, 2009) (Fig. 13). Interestingly, the *Bdnf* level in vehicle treated mice increased as well and there is no significant differences in the level of *Bdnf* between *Rai1*^{+/-} drug and vehicle treated mice ($p = 0.5523$) (Fig. 13). This could potentially be due to the HPCD diluent contained in the vehicle, or could possibly be a result of handling. Further investigation needs to be done without HPCD to narrow down the cause of the increased *Bdnf* expression shown in vehicle treated mice.

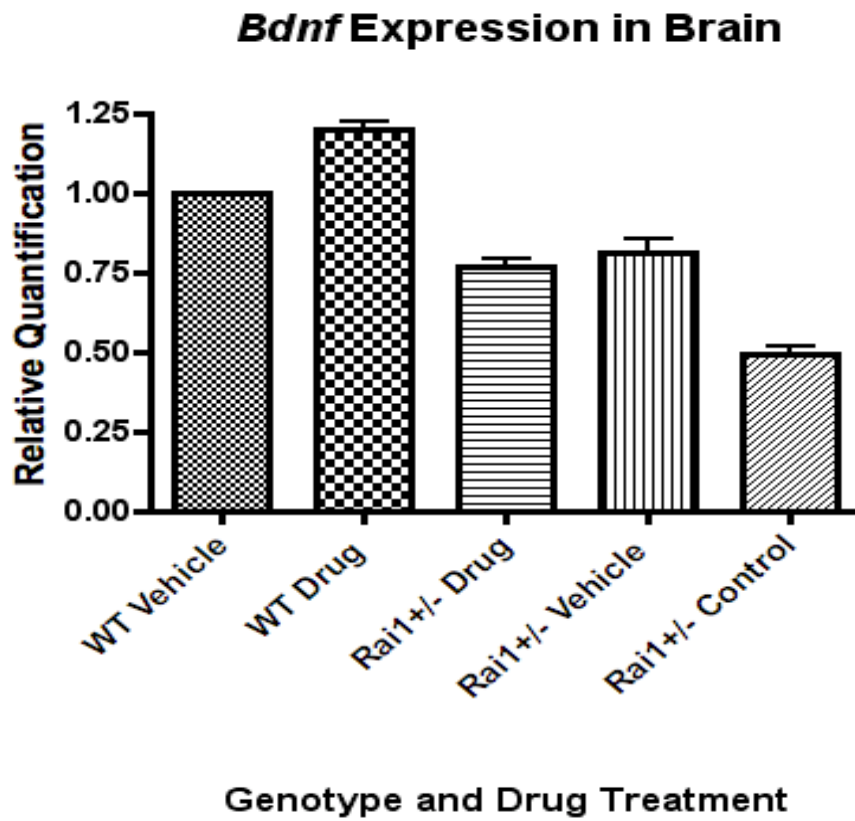


Figure 13. mRNA *Bdnf* expression studies. Results of expression analysis of *Bdnf* in brain tissues without hypothalamus is shown. Data are reported with mean \pm s.e.m. n\$3.

III. Third batch

For the last batch, 12 mice were used. All of these mice were males, of which 6 were drug treated and the other 6 were vehicle. The treatment was continued for 5 days by IP injecting either the drug or vehicle solution. Same drug was used with same concentration and dosage of administration.

Tail flick test.

The tail flick test was performed on the 5th day of treatment. This test gives automated results, thereby yielding more objective data. However, this test is still difficult to perform, as the mouse's body must be held by hand while the tail is exposed in the apparatus. As stated previously, these mixed background mice are very jumpy and hyper, resulting in a number of handling difficulties because the mice kept moving and it was hard to get them still enough to perform the test.

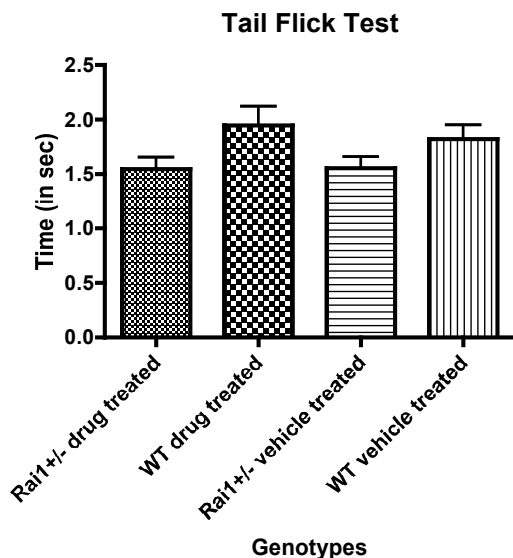


Figure 14. Tail flick test. Tail flick response was observed between the groups. No significant differences were found between any groups. $p=0.3986$

Discussion

For this study, the effect of an ampakine drug, CX1837 on *Rail*^{+/-} mice was evaluated. First, many physical assessments were made to ensure that the drug did not cause any negative side effect or act as a sedative. These tests included sound orienting, pupil constriction/dilation, whisker response, eye blink, ear twitch, postural reflex and response to being picked up by the tail. All mice showed normal responses regardless of genotype or treatment group, suggesting that the drug does not have any negative effects on neurological functioning. This assured that further investigation of this drug was appropriate. However, two of the vehicle treated mice had unexpected death/sickness, which points to a potential confounding effect from the HPCD, which should be removed in the case of future studies.

There are a few things to highlight from this study. First, interesting data was observed in the hot plate test. However, the tail flick test was not successful in capturing changes in the pain response phenotype. I believe this is largely due to the strain of mice being used, as this mixed background FVB/NJ X C57Bl/6J mouse is not an optimal model for assessment of these neurological phenotypes because they are difficult to handle. Evaluation of the pain response has great potential for designing treatments to help with the self-injurious behaviors (i.e. head banging, skin picking) seen with SMS as well as other neurological disorders.

Another effect of the drug seemed to be found in the operant conditioning test. It is very interesting to see that the drug has a significant effect on both WT and *Rail*^{+/-} mice. This makes me wonder if the dosage we used was too high, since the WT drug treated mice showed the effects as well. Different drug dosages can be used to assess this effect to further investigate if this result is a positive or negative attribute related to drug administration.

Growth assessment along with the feeding study is another test to be carefully

considered. While the initial suggested that ampakine treatment has an effect on weight gain and provides a potential solution for obesity, as female *Rai1*^{+/-} treated mice did not gain as much weight as the vehicle treated mice, there were no significant differences found between the treatment and vehicle groups in the second batch. However, it is important to note that the second batch study was only performed for a 2 week period, while the initial study, lasted 8 weeks. Unlike changes in neurological or behavioral phenotypes that can be observed within short timespan, 2 weeks might not have been enough time to observe weight or growth differences, and this result suggests that further investigation needs to be done, with prolonged treatment, to see the growth curve accurately.

Chapter 4. Effect of ampakine treatment on *Bdnf* and whole brain global gene expression

Introduction

Bioinformatic analyses suggest that RAI1 is likely to play a role as a transcriptional regulator; however, its cellular and developmental role is still unclear [15,17]. It has been reported that RAI1 shares stretches of sequence with >50% identity to the transcriptional cofactor TCF20, also known as SPBP [18]. TCF20 also shares >90% of similarity with genomic structure with *RAI1* [15]. The RAI1 protein contains two putative bipartite nuclear localization signals (NLSs), polyglutamine and polyserine tracts, and a PHD/zinc finger domain at the C-terminus [14,15]. *RAI1* mRNA is ubiquitously expressed throughout the tissues, and relative to the other tissues, the heart and brain tissues have stronger expression by two- and threefold, respectively [14]. The alteration in dosage of RAI1 is likely to cause alterations in the expression of different genes downstream. Thus, when *RAI1* is haploinsufficient, *BDNF*, *CLOCK*, *CMKLRI*, and many other genes are altered. Also, two other studies have suggested that RAI1 is likely to be a transcriptional regulator as well [15,18].

BDNF mRNA and protein expression is found throughout the brain with the highest expression in the neurons of hippocampus [58,59,60]. Previously, another student in our lab (B.M. Burns, 2009) has performed microarray analysis on hypothalami and showed reduced in

Bdnf expression in *Rail*^{+/-} mice among many genes. Q-RT-PCR analysis of hypothalamic tissue confirmed that the *Bdnf* level was downregulated by ~2.5 fold in *Rail*^{+/-} mice compared to WT controls [30].

In this chapter, two questions were proposed. First, re-evaluation of *Bdnf* expression in the whole brain of the mice after the drug treatment (without HPCD) was conducted to see if data are consistent with previous findings (Chap. 3). Next, in order to evaluate other genes that are altered due to the CX1837 ampakine drug (restoration of phenotypes as seen in Chap. 3), the whole brain global gene expression was evaluated for each mouse and microarray analysis was used to compare between *Rail*^{+/-} drug vs *Rail*^{+/-} vehicle, as well as between WT drug vs WT vehicle mice.

Materials and Methods

Mouse models, breeding, and maintenance. Mice evaluated in this study were C57Bl/6J *Rail*^{+/-} animals bred from stock received from Jackson Laboratories (stock #005981). Due to poor in-house breeding of this line, the mice were outcrossed to FVB/N to improve number of offspring obtained. When the mice were being bred or in feeding studies, they were given access to a higher fat (9%) Lab Diet 5P06 Prolab RMH 2000 diet and water. An automatic 12-hour light/dark cycle was provided at all times along with constant temperature (21C) and humidity (40%). The overall phenotype of these mice is muted in this mixed background, but the FVB/N x C57Bl/6J *Rail*^{+/-} animals are overweight and have craniofacial anomalies similar to the B6 congenic strain originally evaluated.

Ampakine treatment. Data from the previous study suggested 2-hydroxypropyl- β -cyclodextrin (HPCD) powder was suggested not to be an optimal vehicle. Thus HPCD was removed from this study. An equal mixture of water and 0.9% injectable saline was prepared, and ampakine CX1837 (Cortex Pharmaceuticals) was added to half of the volume to a concentration of 0.25 mg/mL while the remaining half of the saline solution was used as vehicle. The solution containing ampakine CX1837 was then submerged in a sonicator heated to 37C and then frequently vortexed to aid solubilization. The resulting solution is a clear viscous liquid stored in 4C.

Ampakine injection. Each mouse was weighed weekly and given 1 mg/kg BID of a 0.25mg/mL solution of ampakine CX1837 twice daily (10:00 a.m. and 5:00 p.m.). Vehicle solution was used at the same volume for dose of 1 mg/kg. Before the solution was used, it was mixed well to

dissolve any precipitation formed. The volume of drug was altered once a week to account for weight fluctuations in the mice. The mice were anesthetized with isoflurane prior to intraperitoneal (IP) injection. Each mouse received the dose in the same order every day. The four treatment groups included wild type, WT vehicle treated, WT drug treated, *Rai1*^{+/-} vehicle treated, and *Rai1*^{+/-} drug treated.

RNA isolation. RNA was isolated using the Trizol reagent/chloroform method. The quality of isolated RNA was assessed by using a 260/280 and 230/260 absorbency ratio, and the concentration was obtained using Nanodrop.

Real-time quantitative PCR. cDNA synthesis was performed using 4-5 µg of RNA, Invitrogen Superscript 2 reverse transcriptase and random primers according to the manufacturer's instruction. To detect gene expression, for *Bdnf*, an ABI TaqMan probe for mouse mRNA expression was used. All samples were analyzed in triplicate in a 10 µL reaction on an ABI Prism 7500 Fast System with *Gapdh* as an endogenous control.

Microarray. Microarray hybridization was performed in the NARF DNA Microarray Core using an Illumina MouseRef-8 v2.0 expression beadchip. The array was read using an Illumina BeadArray Reader and analyzed by Illumina's GenomeStudio software.

Microarray data Analysis. Using the Illumina GenomeStudio Software, differential analysis was performed between the *Rai1*^{+/-} drug and WT drug vs vehicle. Data were normalized using the quantile method. Differential P-values were determined using the Illumina custom

analysis. The software was also used to calculate a false discovery rate (FDR) and to adjust p-values accordingly. Genes with fold changes were evaluated and top candidate genes were found for further analysis.

Results

***Bdnf* gene expression study in ampakine-treated *Rai1*^{+/-} mice:** For the third round of ampakine study with a total of 12 mice, the treatment was continued for 5 days by IP injection of either the drug or vehicle solution. All mice in this study were males of the same age. Among the 12 mice, 6 were *Rai1*^{+/-} and 6 were WT mice. On the 6th day, before the next injection time, all 12 mice were euthanized and their whole brain tissues were collected. For the RNA extraction, the whole brain tissue was homogenized and 1 mL was taken. The mRNA expression level of *Bdnf* was evaluated to confirm what we had previously found. It is important to note that the vehicle solution in this study was just the mixture of water and saline without HPCD powder, to avoid any of the potential negative effects of HPCD. Ampakine 1837 was only dissolved in water and saline as well.

Fig. 12 shows the results of the *Bdnf* expression studies via qRT-PCR in *Rai1*^{+/-} and WT drug and vehicle treated mice. First, both *Rai1*^{+/-} drug and vehicle treated mice showed an elevation in *Bdnf* level (~up to 80%), and there was no significant difference in levels between the drug and vehicle treated *Rai1*^{+/-} mice ($p=0.5523$) which is consistent with the previous finding (Chap. 3). However, the *Bdnf* level was significantly higher in *Rai1*^{+/-} drug mice ($p=0.0151$), as well as *Rai1*^{+/-} vehicle ($p=0.0281$) mice when compared to the *Rai1*^{+/-} control mice. The control mice are the ones that received neither vehicle nor drug injections. Also, there was no significant difference between WT vehicle and either *Rai1*^{+/-} drug mice ($p=0.0536$) or *Rai1*^{+/-} vehicle mice ($p=0.1564$). One thing that differed in the results of this study was that WT drug treated mice seemed to be unaffected in terms of *Bdnf* expression, consistent with other published reports of ampakine treatment and *Bdnf* expression. Overall, there were no significant

differences between any of the four groups (WT vehicle, WT drug, *Rai1*^{+/-} drug, *Rai1*^{+/-} vehicle) ($p=0.1044$) with regard to expression of *Bdnf*. Therefore, this study suggests that HPCD is not likely to be responsible for the elevation of *Bdnf* level seen in both *Rai1*^{+/-} vehicle and drug treated mice compared to *Rai1*^{+/-} control mice.

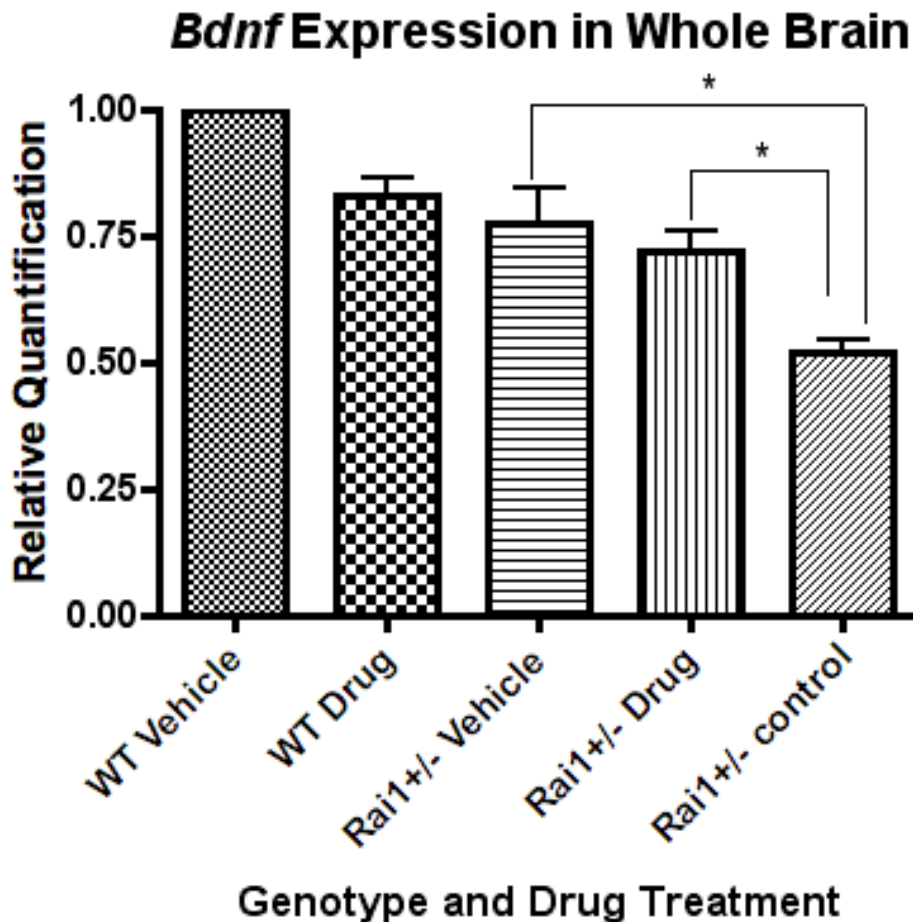


Figure 15. mRNA *Bdnf* expression studies in whole brain. Results of qRT-PCR expression analysis of *Bdnf* in whole brain tissues is shown. * $p \leq 0.05$. Data are reported with mean \pm s.e.m. $n=3$, each sample run in triplicate.

Microarray analysis: The collected RNA from these whole brain samples were also sent out for microarray analysis as described in Materials and Methods. Using the Illumina GenomeStudio Software, two different comparison analyses were made. 1) *Rai1*^{+/-} drug vs. *Rai1*^{+/-} vehicle treated mice and 2) WT drug vs WT vehicle mice, to examine the effects of the drug on the alteration of gene expression. Based on this analysis, the top 5 upregulated and downregulated genes were found by comparing the drug and vehicle groups, with the results listed in Table 1. Table 1 lists the most affected genes in decreasing order of alteration.

First, the top 5 genes that were found to be upregulated due to drug administration in *Rai1*^{+/-} drug mice are as follows: *Olfr1346*, *Wrnip1*, *Bhlhb4*, *Olfr661*, and *Tnfrsf13c* (Table 1a). The top 5 genes that were found to be higher in *Rai1*^{+/-} drug treated mice compared to *Rai1*^{+/-} vehicle are *Nfe2*, *Olfr986*, *Mtmr7*, *Cyp26b1*, and *prl*.

When WT drug and WT vehicle groups were compared, the top five genes that were upregulated due to drug administration include *Vlrc21*, *Evx2*, *Olfr854*, *Gbx1*, and *Trim14*. Likewise, the top five downregulated genes are *Cpa5*, *Flt3*, *Vmn2r28*, *Sec1*, and *Stxbp6*. These top regulated genes include genes involved in the function of DNA binding, genes involved in methyl binding, ATP regulation, transcription regulation, hormones, and olfactory regulation.

In order to understand the possible causes of the pain phenotypes seen from earlier ampakine studies (Chap. 3), what was most interesting to look at was the regulation of any pain related genes. In order to help understand the pain related phenotype we saw with the drug administration, any significant changes in pain related genes were identified within these data. This analysis revealed two genes of interest (Table 2), as described below.

Oncostatin M (*Osm*) showed a 2.8 fold increase in *Rai1*^{+/-} drug treated compared to

Rai1^{+/-} vehicle treated mice. Oncostatin M is a member of the interleukin-6 (IK-6) family of cytokines [78]. These cytokines play important roles in hematopoiesis, inflammation, heart development, and neurogenesis [78]. *Osm*-deficient mice displayed significantly reduced noxious responses in models of acute thermal, mechanical, chemical, and visceral pain [78]. It was noted that *Osm*^{-/-} mice did not exhibit any impairment of motor coordination, yet they showed to have significant reduction in response to various stimuli including mechanical and thermal stimuli [78]. In addition, there was a decrease of a subset of nociceptive DRG neurons in adult *Osm*^{-/-} mice [78]. Increased levels of *Osm* in *Rai1*^{+/-} drug treated mice is interesting to observe as *Rai1*^{+/-} drug treated mice have elevated pain response and *Osm* is responsible for pain sensitivities. Further study needs to be done on this gene in *Rai1*^{+/-} mice and its possible regulation by *Bdnf* and/or *Rai1*.

Another gene of interest was the glutamate (NMDA) receptor, ionotropic, NMDA2D (epsilon 4) (***Grin2d***), which showed a 0.8 fold increase in *Rai1*^{+/-} drug treated mice when compared to *Rai1*^{+/-} vehicle treated mice. It was not surprising to see up-regulation of this gene in the drug treated mice, as the ampakine drug is known for binding to its AMPA receptor and causing an increase in glutamate. NMDAR (receptor for NMADR) has a crucial function in brain development, plasticity and survival [78,79]. Deregulation of these receptors can contribute to a variety of neurological and neurodegenerative disorders [81,82]. This gene also modulates the frequency, rate or extent of the sensory perception of pain, and the series of events required for an organism to receive a painful stimulus, convert it to a molecular signal, and recognize and characterize the signal.

These two genes need to be given special attention in *Rai1*^{+/-} animals that are both untreated with ampakine and untreated in order to determine if this is consistently altered gene in

SMS. Quantitative RT-PCR expression analysis need to be done to confirm the alterations of these genes. Overall, it is important to note that this analysis was done in a very basic manner, and further in-depth analysis is needed. Specifically, researchers should looks further down the list of top regulated genes (i.e. the top ten or more) to see if there are any other genes of interest. These genes should be studied in greater depth to learn more about their functions and roles.

Table 1. Top dysregulated genes found between amphetamine and vehicle treated *Rai1*^{+/-} mice by fold difference. a. Top upregulated genes. b. Top downregulated genes.

a.

Gene Symbol	Fold change	Gene name	Functions
<i>Nfe2</i>	9.6	nuclear factor, erythroid derived 2	Chr15; DNA binding
<i>Olfr986</i>	7.2	olfactory receptor 986	Chr9; olfactory receptor activity
<i>Mtmr7</i>	5.8	myotubularin related protein 7	Chr8; hydrolase activity
<i>Cyp26b1</i>	5.8	cytochrome P450, family 26, subfamily b, polypeptide 1	Chr6; metal ion binding
<i>Prl</i>	5.5	prolactin	Chr 13; hormone activity

b.

Gene Symbol	Fold change	Gene name	Functions
<i>Olfr1346</i>	8.4	olfactory receptor 1346	Chr15; olfactory receptor activity
<i>Wrnip1</i>	6.7	Werner helicase interacting protein 1	Chr13; ATP binding
<i>Bhlhb4</i>	5.6	basic helix-loop- helix family, member e23	Chr 2; Transcription regulated activity
<i>Olfr661</i>	5.1	olfactory receptor 661	Chr7; Olfactory receptor activity
<i>Tnfrsf13c</i>	4.9	tumor necrosis factor receptor superfamily, member 13c	Chr 15; Receptor activity

Table 2. Top dysregulated genes found between amphetamine and vehicle treated WT mice by fold difference. a. Top upregulated genes. b. Top downregulated genes.

a.

Gene Symbol	Fold change	Gene name	Functions
<i>V1rc21</i>	8.3	vomeroneural 1 receptor 4	Chr6; pheromone binding
<i>Evx2</i>	5.9	even skipped homeotic gene 2 homolog	Chr2; DNA binding
<i>Olftr854</i>	5.6	olfactory receptor 854	Chr9; olfactory receptor activity
<i>Gbx1</i>	5.2	gastrulation brain homeobox 1	Chr5; DNA binding
<i>Trim 14</i>	5.2	tripartite motif-containing 14	Chr 4; Metal ion binding

b.

Gene Symbol	Fold change	Gene name	Functions
<i>Cpa5</i>	7.3	carboxypeptidase A5	Chr6; carboxypeptidase activity
<i>Flt3</i>	5.5	FMS-like tyrosine kinase 3	Chr5; ATP binding
<i>Vmn2r28</i>	5.1	vomeroneural 2, receptor 28	Chr 7; G-protein coupled receptor
<i>Sec1</i>	4.4	secretory blood group 1	Chr 7; Fucosyltransferase activity
<i>Stxbp6</i>	4.3	syntrophin binding protein 6	Chr 12; Molecular function

Table 3. Pain related genes and their fold changes. Pain related genes were of particular interest, and the top two upregulated pain-related genes in *Rail* +/- drug were noted.

Gene Symbol	Fold change	Gene name	Process & function
<i>Osm</i>	2.3	oncostatin M	Chr11; response to pain
<i>Grin2d</i>	0.8	glutamate receptor, ionotropic, NMDA2D (epsilon 4)	Chr7; modulates the frequency, rate or extent of the sensory perception of pain

Discussions

In this study, we re-evaluated *Bdnf* expression in mice that were given CX1837 drug and vehicle treatment. This time, HPCD was removed from both the drug and vehicle solutions, due to the negative effects associated with its use, as noted in the previous study. HPCD was originally used because the drug powder is not easily soluble, and HPCD aids solubilization and thus, the delivery of the drug. In this study, the drug was dissolved for a longer period of time, until it formed clear solution. Interestingly, when we evaluated *Bdnf* expression in these sets of mice, the *Bdnf* level was still found to be elevated in the vehicle treated mice (Fig 2). This suggests that HPCD is not the reason for the increase of *Bdnf* expression in vehicle treated mice, and that something else might be going on. These results lead me to ask: 1) Did the *Bdnf* level increase simply because of handling the mice twice daily? or 2) Did it increase because of the sedation of the mice with isofluorine prior to vehicle injection?

It has been reported that “early handling” of rats increases *Bdnf* expression in the rat hippocampus [80]. It is also known that physical exercise is associated with an increased in *Bdnf* mRNA [81]. In one study, it was found that there was a significant, positive correlation between the mean distance run per night and *Bdnf* mRNA in the hippocampus [81]. Also, whisker stimulation and light stimulation increases *Bdnf* mRNA expression in the somatosensory barrel cortex and visual cortex, respectively [82,83].

In order to clarify the question for possible cause of increased in *Bdnf* level in vehicle treated *Rail*^{+/-}, it will be interesting to evaluate mice with just handling itself without injecting. This will help us better understand any possible associations between *Bdnf* expression and animal handling. Thus, in order to clarify the possible cause of an increase in *Bdnf* level in

vehicle treated *Rai1*^{+/-} mice, it would be interesting to evaluate *Bdnf* levels in mice that are handled, but not given injections of any kind, and utilize as controls mice that are not handled at all.

Next, global gene expression analysis was performed on whole brains from mice in this study by microarray using an illumina MouseRef-8 v2.0 expression beadchip. The top candidate genes were found between drug and vehicle treated mice among *Rai1*^{+/-} and WT. The top 5 upregulated genes due to drug administration among *Rai1*^{+/-} mice include *Nfe2*, *Olfir986*, *Mtmr7*, and *Cyp26b1*; however, most interesting to look at were alterations in pain related genes. Two candidate genes were identified. Interestingly, *Osm*^{-/-} mice have been reported to exhibit a significant reduction in pain related responses. This has a direct relationship with the restoration to the pain response seen in the *Rai1*^{+/-} treated mice. *Osm* is potentially one of the genes responsible for this observed phenotype. Thus, this gene will be the top priority gene for further study, as it is likely to be involved in the pain related responses we have observed in drug treated mice. Further analysis is also needed of the other top altered genes to determine how ampakine drug treatment restores phenotypes, connects with SMS phenotypes, and how this drug can be ultimately used as a potential treatment for SMS patients.

Chapter 5. Discussion

After completion of this project, we discovered more about the function of RAI1 and its regulation of *BDNF* and how it connects to SMS phenotypes. First, we confirmed that RAI1 regulates *BDNF* within an intronic region, and this sequence was further narrowed down. This finding is consistent with what was found before using ChIP-chip assay and microarray analysis of *Rai1*^{+/-} mice hypothalami. Next, in order to evaluate the role of *Bdnf*, ampakine drug was utilized in the *Rai1*^{+/-} mouse model. A mouse model is particularly useful when studying a specific gene. Many physical, neurological, and behavioral tests were performed in mice. Significant results were obtained in the hot plate test and operant conditioning test. The hot plate data suggest a potential role of *BDNF* in the SMS phenotype regarding decreased in pain sensitivities. However, because the tail flick test did not capture the pain phenotype observed in the hot plate test, this aspect of the phenotype still needs to be re-investigated with more sophisticated tests.

Unexpected results were found in the gene expression studies via qPCR, which showed an elevation of the *Bdnf* level in vehicle-treated mice. This is inconsistent with what other studies have shown. For example, in the Huntington disease mouse model, ampakine treated mice had higher *Bdnf* levels than those given vehicle in western blot analysis [76]. Also, the study showed that ampakine did not alter *Bdnf* levels in WT mice [76]. However, western blot is not an accurate quantitative measurement. Also, it could be due to use of a different type of

ampakine, as this study used CX929 at a different concentration than our study.

From the data shown here study, it is important to note that any significant findings can be due to other factors or genes that were caused by drug administration. Also, after completion of this study, one has to consider other means by which the expression level of *Bdnf* might increase. Could daily handling of mice alter the *Bdnf* level? Does administration of anesthetic alter *Bdnf*? More evaluations and further study of possible variables (i.e. drug concentration, animal handling, etc.) need to be done to discover more about this ampakine drug and its effects.

Lastly, *Bdnf* levels were compared between the *Rai1* +/- drug and vehicle treated mice without HPCD diluent. Interestingly, the vehicle mice still had elevated *Bdnf* level and there was no differences between the *Rai1* +/- drug and vehicle treated with regard to expression of *Bdnf*. Also, microarray analysis was performed on whole brain to evaluate the global expression analysis and top candidate genes that were altered due to drug administration were discovered. The top 5 genes that were altered due to administration were identified to be *Olfr1346*, *Wrnip1*, *Bhlhb4*, *Olfr661*, and *Tnfrsf13c*. Also, considering pain restoration phenotype found due to drug administration, any pain related genes were evaluated. Two candidate genes *Osm*, and *Grin2d* were identified. Increased level of *Osm* in *Rai1* +/- drug treated mice is interesting to observe as *Rai1* +/- drug treated mice have elevated response and *Osm* is responsible for pain sensitivities.

Overall, these studies opened up many other areas to explore regarding *Bdnf* levels, mouse behavior, and ampakine drugs. Therefore, I suggest future directions for each aim below.

Future directions for Aim 1.

We have confirmed that *RAI1* has a direct binding sequence in the *BDNF* intronic region, and we have further narrowed down its sequence to ~800 bp. For the future, further narrowing

down within this region would be a logical follow-up. Starting with *BDNF short^{Luc}*, even shorter pieces can be created and the relative luciferase activities examined until there is no more alteration in luciferase activity observed; thus, identifying the exact consensus binding sequence for RAI1. I think the remaining RAI1 binding sequence found in the ChIP-ChIP should be removed last, as it will be very informative to know that ChIP-ChIP analysis is actually showing the accurate binding peak. From this suggested study, we can ultimately find a consensus binding sequence.

Next, it will be also interesting to take the sequence that was removed from the original *BDNF^{Luc}*, thereby creating another short form of *BDNF* to see if RAI1 also regulates *BDNF* expression via this region. It is still possible to see some regulation, as potential repression or additional enhancer regions can lie within the sequence.

It is also important to study the pathway downstream from *BDNF*, as this will further narrow down the specific role of *BDNF*, as many different roles of *BDNF* have been found, as previously stated.

Future directions for Aim 2.

Because the tail flick test did not capture the pain phenotype previously observed in the hot plate test, this aspect of the phenotype still needs to be re-investigated. More sophisticated tests need to be done to better assess the pain sensitivity experienced by the ampakine treated mice. Options include incision prior to ampakine injection and/or lactic acid injection [84,85].

Another possibility that is more costly but potentially a better option in terms of long term testing is to obtain a different strain of mice that are known for having calmer behavioral characteristics. If the mice are calmer and easier to handle, not only can pain sensitivity be re-

evaluated, but other tests could be added that might capture other phenotypes not seen in this study. Also, the consequences of drug administration can be more easily observed.

Furthermore, another study could be done by altering the ampakine dosage or by using different types of ampakine, such as CX1739. For this study described here, the maximum suggested dosage was used. Injecting a smaller amount of the drug would allow the minimum dosage needed for effect to be observed, which would be useful in evaluating ampakines as a potential treatment for SMS and other syndromes with reduced pain sensitivity. It would be helpful to know the minimum dosage for oral administration. Also, examining different dosages can potentially help us find an even better dosage that works more effectively in these mice.

Future directions for Aim 3.

Using a microarray to evaluate expression of genes in the whole brain of drug treated and vehicle treated WT and *Rai1*^{+/-} mice, we found top candidate genes that are altered due to drug administration. Also, pain related genes were identified. Direct follow up from this study would be to take these candidate genes and perform Q-PCR to confirm the true alterations in the expression. Furthermore, as we now have microarray data from both the whole brain and hypothalamus (B.M. Burns, 2009) of the mice, we can compare these data and analyze to see if there are any genes that are commonly and differently expressed within these tissues.

At last, use of a pure strain for any additional studies to assess drug effects would minimize background noise in the microarray data. Further, specific regions of the brain, such as hippocampus, which is the tissue that *Bdnf* is most highly expressed, can be used.

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